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CONTRACTING ORGANIZATION: Duke University Medical Center
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13. SUPPLEMENTARY NOTES

14. ABSTRACT
We provide the first evidence that Snail-1, a tumor progression factor[1-3], influences breast tumor cell adhesion to matrix proteins. Specifically, we show that Snail-1 reduces alpha2 beta1 integrin expression levels in breast tumor cells. This novel Snail-1 activity results in impaired tumor cell adhesion to the alpha2 beta1 integrin-binding matrix protein Collagen-1. As a specificity control, we show that Snail-1 does not influence alpha5 beta1 integrin levels or alpha5 beta1 integrin-mediated adhesion to Fibronectin. Previous studies indicate a suppressive role for the alpha2 beta1 integrin in breast tumor cell migration[4, 5]. Accordingly, we postulate that Snail-1 repression of alpha2 beta1 integrin expression promotes breast tumor cell motility, a topic of current study. Based on the knowledge that tumor cell migration is critical for tumor metastasis, we postulate that this novel Snail-1 function promotes tumor metastasis, an area for future investigation.

15. SUBJECT TERMS
Snail; integrin; breast cancer; migration; adhesion

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INTRODUCTION: This work examines a novel function for Snail-1, a transcriptional inhibitor implicated in tumor metastasis[1-3], in the regulation of alpha2 beta1 integrin expression in breast tumor cells. We address the hypothesis that Snail-1 represses alpha2 integrin expression in metastatic breast carcinoma cells. This hypothesis is grounded on the knowledge that the alpha2 promoter contains four consensus Snail-1 binding sites (Bachelder, unpublished observation), and that other Snail-1 family members have been shown to impede integrin transcription in keratinocytes[6]. The alpha2 integrin subunit pairs exclusively with beta1 integrin subunits, and alpha2 is limiting for alpha2 beta1 heterodimer assembly. Accordingly, we examine the impact of Snail-1 on alpha2 beta1 integrin levels in breast tumor cells, as well as the ability of these cells to adhere to Collagen-1, an alpha2 beta1-binding matrix protein. Based on published work indicating that alpha2 beta1 integrin represses breast tumor cell migration[4, 5], we hypothesize that Snail-1 stimulates breast tumor cell migration by reducing alpha2 beta1 integrin levels. Collectively, these studies investigate if alpha2 beta1 integrin is a novel target of Snail-1 transcriptional inhibition, and examine if Snail-1 regulation of this novel target in breast tumor cells promotes tumor cell motility. Since tumor cell migration is a requirement for metastasis, we predict that this Snail-1 activity is relevant to breast cancer metastasis, a topic for future studies.

BODY:

Task #1: Examine if the alpha2 beta1 integrin is a novel target of Snail-1 activity. Our initial studies did not demonstrate an effect of Snail-1 on beta1 integrin subunit expression levels in breast tumor cells (see Supporting Data, Fig. 3). Consequently, we examined the ability of Snail-1 to regulate expression of alpha2, an integrin subunit that associates with the beta1 integrin subunit to produce alpha2 beta1 heterodimers. The rationale for this focus includes the knowledge that: 1) the alpha2 promoter contains four consensus Snail-1 binding sites (Bachelder, unpublished observation), 2) the alpha2 subunit pairs exclusively with beta1 subunits to produce alpha2 beta1 integrin heterodimers, 2) previous studies indicate an ability of the alpha2 beta1 integrin to suppress breast tumor cell migration[4, 5]. The first step in this study was to establish a model for detecting endogenous Snail-1 protein in breast tumor cell lines. We tested the ability of several commercial Snail-1 antibodies for their ability to detect Snail-1 protein in extracts from breast cancer cell lines. However, none of these antibodies was sufficiently sensitive for this purpose (data not shown). Next, we tested the ability of a monoclonal Snail-1 antibody from our collaborator, Dr. Antonio Garcia de Herreros, to detect Snail-1 in protein extracts from breast tumor cells. In Fig. 1A (see Supporting Data), we demonstrate by immunoblotting that this antibody detects Snail-1 protein in extracts from two metastatic human breast cancer cell lines, but not in an extract from human mammary epithelial cells, which lack endogenous Snail-1[7]. We also developed an effective immunohistochemistry protocol for detecting Snail-1 in primary breast tumors using this monoclonal antibody(Fig. 1B).

We next sought to establish an Snail-1 siRNA transfection protocol for reducing Snail-1 expression levels in breast tumor cell lines. In Fig. 2A (Supporting Data), we demonstrate that Snail-1 mRNA expression levels are reduced significantly in MDA-MB-231 transfected with a Snail-1 siRNA (50 nM) 8 hours post-transfection. We next examined Snail-1 protein levels in MDA-MB-231 at two times following their transfection with the Snail-1 siRNA. As shown in Fig. 2B, Snail-1 protein was reduced only 1.6-fold 8 hours post-transfection, but 4.9-fold 40 hours post-transfection. Accordingly, we chose to assess the effects of Snail-1 siRNA transfection on alpha2 beta1 integrin expression 40 hours post-transfection.

Using this siRNA transfection protocol, we next investigated the effect of reducing Snail-1 expression in MDA-MB-231 breast tumor cells on the levels of the alpha2 integrin subunit, which pairs exclusively with beta1 subunits to produce alpha2 beta1 integrin heterodimers. As shown in Fig. 3 (Supporting Data), reducing Snail-1 expression in these cells resulted in increased alpha2 protein levels. As a control for specificity, we showed that alpha5 integrin levels were not increased in Snail-1 siRNA transfectants.

During the six month extension period of this project, we are examining if Snail-1 regulates alpha2 expression by suppressing its transcription. These studies have been delayed because: 1) it took some time to find a collaborator with an alpha2 integrin promoter-driven reporter construct, and 2) our laboratory technician, who was pursuing these studies, accepted another position after two months of working on this project. Currently, a graduate student (Hsin-Ying Chen) is devoting 100% effort to this project. We have obtained an alpha2 promoter-driven luciferase construct from our collaborator, Dr. Thomas Kunicki (The Scripps Research Institute), and are examining the impact of Snail-1 on alpha2 promoter activity using this construct. In addition, Hsin-Ying is optimizing a real time PCR protocol to measure alpha2 mRNA levels in breast tumor cell lines. It is anticipated that we will complete our assessment of Snail-1 regulation of alpha2 transcription in this 6 month extension period.

Task #2: Determine the regulation of breast tumor cell adhesion, migration, and survival by Snail-1. We examined the impact of reducing Snail-1 expression in MDA-MB-231 breast tumor cells on their adhesion to matrix proteins. Considering that the major ligand of the alpha2 beta1 integrin is Collagen-1[8], we focused our studies on Snail-1 regulation of breast tumor cell adhesion to Collagen-1. As shown in Fig. 4 (Supporting Data), Snail-1 siRNA transfected MDA-MB-231 cells exhibit a significantly increased ability to bind to Collagen-1 compared to control transfectants. As a specificity control, we showed that the ability of MDA-MB-231 cells to bind to Fibronectin, an alpha5 beta1 integrin-binding matrix protein, was not influenced by Snail-1 siRNA transfection (Fig. 4). In the six month extension period of this project, we are examining effects of the Snail-1/alpha2 integrin signaling axis on breast tumor cell migration and survival.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a protocol for detecting endogenous Snail-1 protein levels in breast tumor cells using a Snail-1 monoclonal antibody from our collaborator, Dr. Antonio Garcia de Herreros (commercially-available antibodies were not of sufficient sensitivity).
- Established a Snail-1 siRNA transfection protocol for effectively reducing Snail-1 protein levels in MDA-MB-231 breast tumor cells
- Established an immunohistochemistry protocol for detecting Snail-1 protein in human breast tumors
- Demonstrated that Snail-1 suppresses breast tumor cell adhesion to Collagen-1, an alpha2 beta1 integrin binding matrix protein, but not to Fibronectin, an alpha5 beta1 integrin binding matrix protein
- Established that Snail-1 reduces alpha2 integrin subunit, but not alpha5 integrin subunit expression in breast tumor cells

REPORTABLE OUTCOMES:

- 2008 Era of Hope Meeting (Baltimore, MD): "Novel Activity for Snail-1 in integrin regulation"
- Preliminary data for R21 grant submission: "Nuclear Snail-1 in the progression of Estrogen Receptor(-) invasive breast cancer"

CONCLUSION: This work is the first to implicate Snail-1 in the regulation of breast tumor cell adhesion. We describe a novel target of Snail-1 (alpha2 integrin) in breast tumor cells. We show that Snail-1 suppression of alpha2 integrin expression results in reduced tumor cell adhesion to the alpha2 beta1 integrin-binding matrix protein, Collagen-1. During the six month extension period granted for this project, we will examine if Snail-1 impacts alpha2 integrin levels in breast tumor cells by directly suppressing alpha2 integrin subunit transcription. We will validate our *in vitro* findings in primary tumors, examining if Snail-1 predicts reduced alpha2 integrin expression in human breast cancers. We will also examine the impact of the Snail-1/alpha2 integrin signaling axis on breast tumor cell motility. Considering that tumor cell motility is an important requirement for metastasis, it is anticipated that these studies will ultimately result in the identification of novel therapeutic targets for impeding breast tumor metastatic progression.

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10. Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM: **Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial-mesenchymal transition**. *J Cell Biol* 2005, **168**(1):29-33.
11. Pan H, Wanami LS, Dissanayake TR, Bachelder RE: **Autocrine semaphorin3A stimulates alpha2 beta1 integrin expression/function in breast tumor cells**. *Breast cancer research and treatment* 2008.

APPENDICES:

CURRICULUM VITAE

Date prepared: September 28, 2008

Name: Robin Elizabeth Bachelder, Ph.D.

Primary academic appointment: Pathology

Primary academic department (not DUAP):

Secondary appointment (if any) - (department):

Present academic rank and title: Assistant Professor of Pathology

Date and rank of first Duke faculty appointment: September, 2005- Assistant Professor

Date of birth: April 22, 1968 **Place:** Springfield, MA

Citizen of: U.S.

Visa status (if applicable):

Education:	**Institution**	**Date**	**Degree**
College	Colgate University Hamilton, New York	1990	B.A.
Graduate School or Professional School	Harvard Medical School Division of Medical Sciences Committee on Virology	1996	Ph.D.

Professional training and academic career (chronologically, beginning with first postgraduate position):

Institution	Position/Title	Dates
Harvard Medical School Boston, MA	Research Fellow Department of Pediatric Oncology Laboratory of Dr. Steven Burakoff	1996-1997
Harvard Medical School Boston, MA	Research Fellow in Medicine Laboratory of Dr. Arthur Mercurio	1997-1999
Harvard Medical School Boston, MA	Instructor in Medicine Laboratory of Dr. Arthur Mercurio	1999-2005

PUBLICATIONS:

Refereed Journals:

1. Pan, H., Wanami, L.S., Dissanayake, T.R., and Bachelder, R.E. 2008. Autocrine semaphorin3A stimulates alpha2 beta1 integrin expression/function in breast tumor cells. Breast Cancer Res. Treat. In Press.

2. Wanami, L.S., Chen, H., Peiro, S., Garcia de Herreros, A., and Bachelder, R.E. 2008. Vascular endothelial growth factor-A stimulates Snail expression in breast tumor cells: Implications for tumor progression. Exp. Cell Res. 314: 2448-53.

3. Folgiero, V., Bachelder, R.E., Bon, G., Sacci, A., Falcioni, R., and Mercurio, A.M. 2007. The alpha 6 beta 4 integrin regulates ErbB-3 translation: Implications for alpha 6 beta 4 signaling and function. Cancer Res. 67:1645-52.

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Non-Refereed Journals:

1. Mercurio, AM, Lipscomb, EA and Bachelder, RE. 2005. Nonangiogenic Functions of VEGF in Breast Cancer. Journal of Mammary Gland Biology and Neoplasia 10: 283-290.

2. Mercurio AM, Bachelder RE, Bates RC, and Chung J. 2004. Autocrine signaling in carcinoma: VEGF and the a6b4 integrin. Seminars in Cancer Biol. 14: 115-122.

3. Mercurio AM, Bachelder RE, Chung J, O'Connor KL, Rabinovitz I, Shaw, LM and Tani T. 2001. Integrin laminin receptors and breast carcinoma progression. J. Mammary Gland Biol. Neoplasia 6: 299-309.

4. Mercurio AM, Bachelder RE, Rabinovitz I, O'Connor KL, Tani T, and Shaw LM. 2001. The metastatic odyssey: the integrin connection. Surg. Oncol. Clin. N. Am. 10: 313-328.

5. Bachelder RE, Letvin NL. 1996. Postbinding functions of CD4 in HIV infection. Trends in Microbiology 4: 359-363.

Projects/Research Interests:

Direct effects of VEGF antagonists on tumor cells

Regulation of PTEN tumor suppressor activity by VEGF receptors in tumor cells

Identification of novel activities of Snail-1 in breast cancer

Proceedings of Meetings:

1. Chen, H.S. and Bachelder, R.E. 2008. Novel activity for Snail in integrin regulation. Era of Hope Meeting, Department of Defense, Breast Cancer Research Program.

2. Wanami, L. and Bachelder, R.E. 2008. The Vascular Endothelial Growth Factor regulates Snail expression in breast tumors: Implications for tumor progression. American Association for Cancer Research.

3. Pan, H., Wanami, L., and Bachelder, R.E. 2007. Semaphorin3A increases expression in breast tumor cells of the a2b1 integrin: a cell migration inhibitor. American Association for Cancer Research, Advances in Breast Cancer.

4. Bachelder RE, Lipscomb E, Wendt MA, Eickholt BJ, and Mercurio AM. 2003. Semaphorin 3A is an endogenous suppressor of breast carcinoma chemotaxis/invasion. Keystone Symposium. Cell Migration and Invasion.

5. Bachelder RE, and Mercurio AM. 2000. Crosstalk between Fas apoptotic and AKT survival signaling pathways: Regulation by integrins. Programmed Cell Death Regulation: Basic mechanisms and therapeutic opportunities. American Association for Cancer Research.

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8. Bachelder RE and Letvin NL. 1995. Characterization of CD4-specific Fabs from a recombinant Fab library generated from a human recombinant soluble CD4-immunized, HIV-1-infected human. Molecular approaches to the control of infectious diseases. Cold Spring Harbor Press.

Invited Oral Presentations:

2008 Duke University Medical Center, Department of Medicine, Laboratory of Dr. Victoria Seewaldt- "Identification of novel breast tumor therapeutic targets"

2007 Duke University Medical Center, Department of Radiation Oncology, Laboratory of Dr. Mark Dewhirst. "Glycogen synthase kinase in breast cancer"

2007 Duke University Medical Center, Department of Medicine, Laboratory of Dr. Gerard Blobe. "Autocrine regulators of the epithelial-mesenchymal transition"

2007 Duke University Medical Center, Pathology Graduate Student Seminar "Beyond Angiogenesis: Autocrine functions for VEGF in tumor cells"

2006 Duke University Medical Center, Grand Rounds. "Crossing Disciplines: What breast cancer researchers can learn from neurobiologists"

2005 Duke University Medical Center, Grand Rounds. "Beyond Angiogenesis: Autocrine functions for VEGF in breast carcinoma progression".

2003 Dana Farber Cancer Institute, Boston, MA. Harvard Cancer Center Cell Migration Meeting. "Regulation of carcinoma cell migration by alternative neuropilin-1 ligands".

2003 Beth Israel Deaconess Medical Center, Division of Cancer Biology and Angiogenesis Seminar Series, Boston, AM. "Autocrine regulation of carcinoma cell survival and invasion".

2003 Dana Farber Cancer Institute, Boston, MA. Dana Farber SPORE seminar series "VEGF autocrine signaling as a potential target for breast cancer therapy".

Professional awards and special recognitions:

2007	Department of Defense, Breast Cancer Research Program Concept Award
2006-2007	Duke Comprehensive Cancer Center Pilot Project Award
2005	Department of Defense Breast Cancer Research Program Study Section (Pathobiology 3)
2002-2007	NCI Howard Temin Research Scientist Development Award (K01)
2000-present	Director, Flow Cytometry shared resource, Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center
2000-present	Group leader, VEGF Meeting, Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center
2003	Department of Defense Breast Cancer Research Program Study Section (Pathobiology 3)
2003	Dana Farber/Harvard Specialized Program of Research Excellence Center Development Award
1998-1999	National Research Service Award, Alpha6-Beta4 Integrin Regulation of Carcinoma Survival
1996-1997	Center for AIDS Research Grant Award, Dana Farber Cancer Institute, HIV-induced T lymphocyte signaling
1993-1995	Ryan Fellowship for Outstanding Achievement in Medical Sciences
1990-1992	National Research Service Award, National Institutes of Health

Committees and Administrative Activities:

2008 Thesis committee- Kelly Kennedy, graduate student in the laboratory of Dr. Mark Dewhirst, Department of Pathology

2008 Duke University, Department of Pathology. Interviewed graduate student applications.

2007 Thesis committee- Kelly Gordon, graduate student in the laboratory of Dr. Gerard Blobe, Department of Medicine

2007 Duke University School of Medicine. Interviewed medical student applicants.

2007 Duke University, Department of Pathology. Interviewed graduate student applicants.

Teaching and Mentoring:

2008 Instructor in Molecular Aspects of Disease (PTH385), Duke University School of Medicine

2007 Instructor in Molecular Aspects of Disease (PTH385), Duke University School of Medicine

External Support:

Current

Department of Defense
9/01/07-2/28/09 (including a six month extension)
Bachelder (PI)
Influence of Snail on beta1 integrin expression/activity in breast carcinoma
The novel idea that the alpha2 beta1 integrin is a target of Snail activity in breast carcinoma is examined.

Completed

K01 CA093855-06
7/01/02-6/30/08
Bachelder (PI)
National Cancer Institute
Novel Function for VEGF in Breast Carcinoma
This study investigates novel autocrine functions for VEGF in regulating the survival and chemotaxis of breast carcinoma cells.

Duke Comprehensive Cancer Center Pilot Award
7/01/06-6/30/07
Bachelder (PI)
Autocrine Regulation of Snail, a Breast Cancer Progression Factor

PAID PERSONNEL:

Luke S. Wanami, Research Technician
Hsin-Ying Chen, Graduate Student
Robin Bachelder, Ph.D., Principal Investigator

Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial–mesenchymal transition

Robin E. Bachelder,[1] Sang-Oh Yoon,[1] Clara Franci,[2] Antonio García de Herreros,[2] and Arthur M. Mercurio[1]

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We report that the activity of glycogen synthase kinase-3 (GSK-3) is necessary for the maintenance of the epithelial architecture. Pharmacological inhibition of its activity or reducing its expression using small interfering RNAs in normal breast and skin epithelial cells results in a reduction of E-cadherin expression and a more mesenchymal morphology, both of which are features associated with an epithelial–mesenchymal transition (EMT). Importantly, GSK-3 inhibition also stimulates the transcription of Snail, a repressor of E-cadherin and an inducer of the EMT. We identify NFκB as a transcription factor inhibited by GSK-3 in epithelial cells that is relevant for Snail expression. These findings indicate that epithelial cells must sustain activation of a specific kinase to impede a mesenchymal transition.

Introduction

The distinct architecture of epithelia is derived from the interactions of epithelial cells with each other, with the underlying basement membrane, and, less directly, with mesenchymal cells in the stroma. Within this complex set of interactions, a prime determinant of epithelial structure is E-cadherin, a transmembrane protein that mediates Ca^{++}-dependent, homophilic intercellular adhesion (Thiery, 2002; Nelson and Nusse, 2004). The central role of E-cadherin in epithelia is evidenced by the fact that loss of either its expression or function results in the dissolution of the epithelial architecture and the acquisition of a mesenchymal phenotype. This process, referred to as the epithelial–mesenchymal transition (EMT), occurs in the contexts of development and tumor progression (Thiery, 2002).

The central importance of E-cadherin for epithelial architecture leads to the novel hypothesis that epithelial cells may support signaling pathways that preserve E-cadherin expression as a means of preventing an EMT. In this connection, we were intrigued by reports that glycogen synthase kinase-3 (GSK-3), a ubiquitously expressed protein serine kinase, is active in resting epithelial cells (Papkoff and Aikawa, 1998; Murray et al., 1999), but that its function in epithelial biology had not been defined. Our assessment of GSK-3 function in epithelial cells revealed that its activity is essential for maintaining epithelial structure because it maintains the expression of E-cadherin. Inhibition of GSK-3 activity or expression results in a bona fide EMT. Moreover, we report that one mechanism by which GSK-3 maintains E-cadherin expression is by inhibiting the transcription of Snail, a zinc finger transcriptional repressor of E-cadherin that is absent in epithelial cells but expressed in tumors (Batlle et al., 2000; Cano et al., 2000; Blanco et al., 2002).

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Abbreviations used in this paper: EMT, epithelial–mesenchymal transition; GSK-3, glycogen synthase kinase-3; siRNA, small interfering RNA.

Results and discussion

Initially, we assessed the effects of inhibiting GSK-3 activity in normal breast epithelial cells (MCF10A) using SB415286, a highly specific, small molecule inhibitor of GSK-3 (Coghlan et al., 2000). The ability of SB415286 to inhibit GSK-3 activity was evidenced by the increased levels of cyclin D1, a protein subject to GSK-3–dependent proteolysis (Diehl et al., 1998), in SB415286-treated MCF10A cells, relative to those treated with DMSO (Fig. 1 A). Inhibiting GSK-3 activity also disrupted the epithelial morphology of these cells, as evidenced by the loss of cell–cell contacts (Fig. 1 A).

Loss of E-cadherin and expression of mesenchymal proteins are defining steps in the EMT. Based on our observation that inhibition of GSK-3 activity reduced cell–cell contacts in MCF10A epithelial cells, we hypothesized that GSK-3 may be a regulator of E-cadherin expression and an inhibitor of the EMT. Supporting these hypotheses, the treatment of MCF10A

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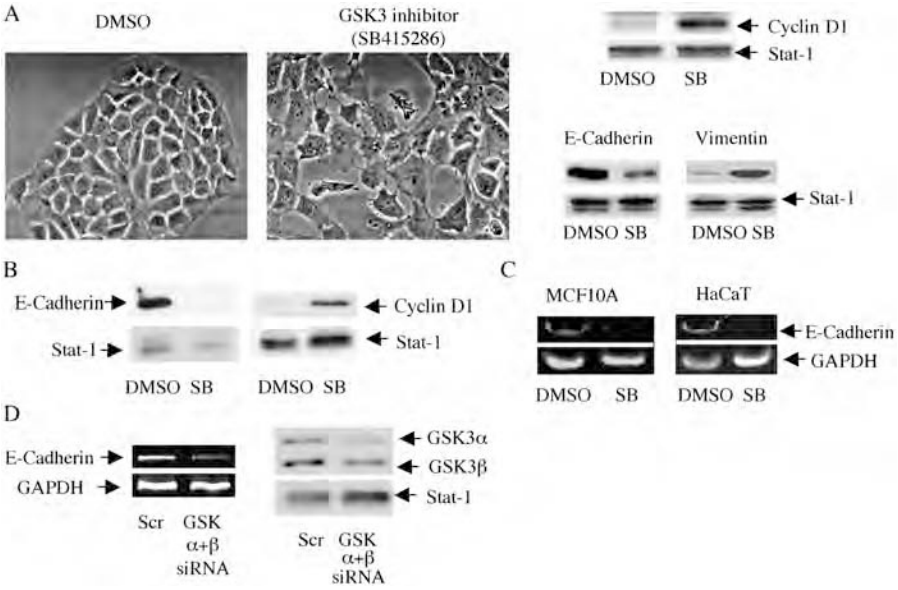

Figure 1. **GSK-3 maintains the epithelial phenotype.** (A) MCF10A cells were incubated with either DMSO or 25 μM SB415286 (Biosource International) in 0.5% FBS-containing medium. Cell morphology was assessed after 72 h by phase contrast microscopy. Expression of Cyclin D1, Stat-1, E-cadherin, and vimentin was assessed by immunoblotting. (B) HaCaT cells were incubated with DMSO or SB415286, and E-cadherin, Stat-1, and cyclin D1 expression was assessed as indicated in A. Similar results were obtained in four independent experiments. (C) E-cadherin and GAPDH mRNA levels were determined after 48 h of drug treatment by RT-PCR. (D) MCF10A cells were cotransfected with GSK-3α– and GSK-3β–specific inhibitory RNA pools (α+β siRNA) or a nonspecific control pool (Scr). RNA was extracted from these cells 48 h after transfection, and E-cadherin and GAPDH mRNA levels were determined by RT-PCR. GSK-3 and Stat-1 expression was assessed by immunoblotting.

cells with SB415286 reduced the expression of total cellular E-cadherin protein significantly and induced the expression of the mesenchymal protein vimentin (Fig. 1 A) without influencing cell viability (not depicted). Together, these data indicate that epithelial cells in which GSK-3 activity has been inhibited manifest changes characteristic of an EMT.

The effects of GSK-3 inhibition can be generalized to other epithelial cells, as demonstrated by the significantly reduced levels of E-cadherin protein in HaCaT skin cells that had been incubated with the GSK-3 inhibitor (Fig. 1 B). This treatment also increased cyclin D1 expression, indicating the efficacy of SB415286 in HaCaT cells (Fig. 1 B). GSK-3 inhibition also reduced the levels of E-cadherin mRNA in GSK-3 inhibitor–treated HaCaT and MCF10A cells, relative to controls (Fig. 1 C). We implemented a small interfering RNA (siRNA) strategy to reduce expression of both the α and β isoforms of GSK-3 in MCF10A cells. Cells were transfected with a pool of eight siRNAs, targeting unique regions of GSK-3α and GSK-3β genes, or with a pool of nonspecific sequences (Fig. 1 D, Scr). Relative to the control pool, the GSK-3α and GSK-3β siRNA pool significantly decreased expression of GSK-3α and GSK-3β proteins (Fig. 1 D). Similar to the effect of the GSK-3 inhibitor, GSK-3–specific siRNAs markedly reduced E-cadherin mRNA (Fig. 1 D).

We next sought to determine the mechanism by which GSK-3 regulates E-cadherin expression. Snail, a member of the zinc finger family of transcriptional repressors, is an established suppressor of E-cadherin transcription, and its activity is an important determinant of the EMT, in the contexts of both mesoderm development (Carver et al., 2001) and tumor progression (Batlle et al., 2000; Cano et al., 2000). Snail is absent in epithelial cells but expressed in tumors, and its expression has been shown to correlate inversely with tumor grade (Blanco et al., 2002). We investigated whether GSK-3 regulates E-cadherin expression by repressing Snail expression. Inhibition of GSK-3 activity in either MCF10A or HaCaT cells significantly increased Snail mRNA levels (Fig. 2 A). Snail mRNA levels were also elevated in epithelial cells transfected with GSK-3–specific siRNAs (Fig. 2 A). As evidence that GSK-3 inhibition alters Snail transcription, we observed increased activity of a Snail promoter–driven reporter gene in HaCaT cells treated with SB415286, relative to that measured in DMSO-treated cells (Fig. 2 B). Increased levels of Snail protein were also detected in cells treated with the GSK-3 inhibitor (Fig. 2 C).

Because of our recent finding that NFκB drives Snail expression (Barbera et al., 2004), we explored whether GSK-3 inhibits Snail transcription by repressing NFκB activity. Indeed, inhibition of GSK-3 activity in HaCaT cells stimulated NFκB-dependent reporter gene expression (Fig. 3 A). In addition, we observed significantly decreased levels of IκB, an inhibitor of NFκB, in SB415286-treated MCF10A cells (Fig. 3 B). Finally, an NFκB inhibitor (SN50) suppressed the ability of SB415286 to induce Snail expression (Fig. 3 C). Together, these data indicate that the NFκB pathway is inhibited by GSK-3 in epithelial cells, which results in the silencing of Snail expression.

The data presented here indicate that GSK-3, a kinase that is active in resting epithelial cells (Papkoff and Aikawa, 1998; Murray et al., 1999), is a critical determinant of epithelial structure and a suppressor of the EMT. This finding implies that epithelial cells must sustain activation of a specific kinase to prevent an EMT, a mechanism distinct from that suggested by previous studies, which have shown that activation of kinases such as Akt (Grille et al., 2003) and ILK (Oloumi et al., 2004) can promote an EMT. An important implication of our findings is that endogenous suppressors of GSK-3, such as Wnt and PI3-kinase, which are frequently activated in carcinoma cells (Woodgett, 2001), may also inhibit E-cadherin transcription and promote an EMT. Our observation that inhibition of both GSK-3α and GSK-3β isoforms increased Snail expression in epithelial cells (Fig. 2 A), whereas inhibition of either isoform alone had no effect on Snail levels (not depicted), indicates that these isoforms may serve redundant functions in maintaining epithelial architecture. Thus, we hypothesize that

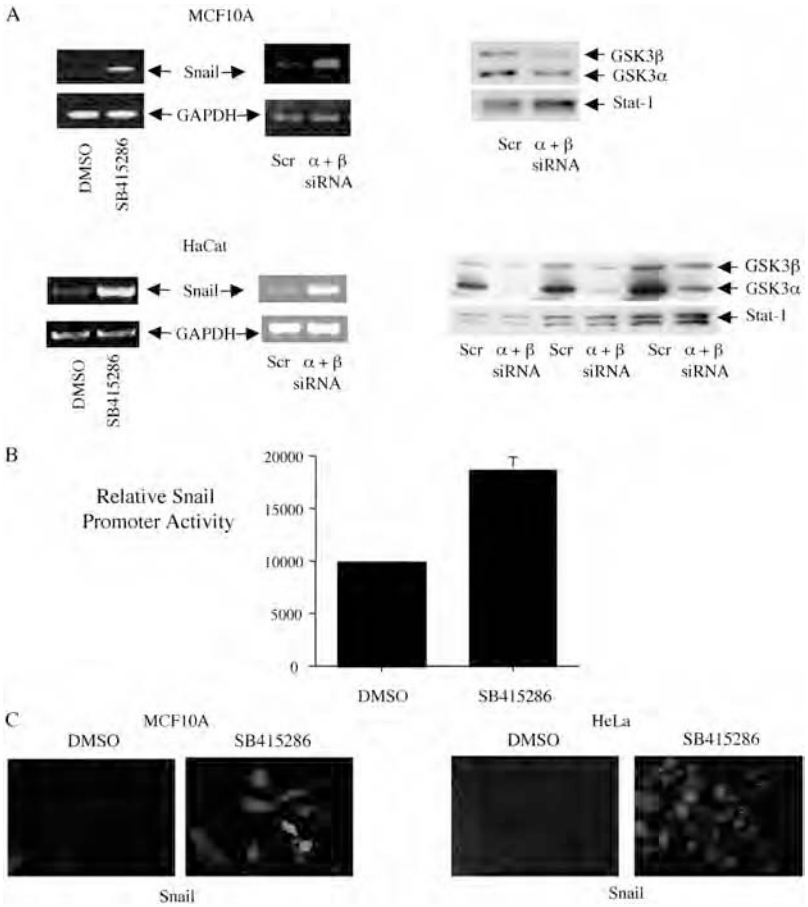

Figure 2. **GSK-3 inhibits Snail transcription in epithelial cells.** (A) MCF10A and HaCaT cells were either incubated with 25 μM of the GSK-3 inhibitor SB415286 or with DMSO in 0.5% FBS-containing medium. Alternatively, these cells were transfected with GSK-3α– and GSK-3β–specific inhibitory RNA pools (α+β siRNA) or a nonspecific control pool (Scr). Snail and GAPDH mRNA levels were assessed by reverse transcription, followed by PCR. GSK-3 and Stat-1 expression was assessed by immunoblotting. (B) HaCaT cells were cotransfected with a Snail promoter–driven and a control β-galactosidase reporter construct. These transfectants were then incubated with DMSO or SB415286, as described in A, for 24 h. Data are reported as relative luciferase activity (normalized to β-galactosidase activity), ± SD, and are representative of three independent experiments. (C) MCF10A and HeLa cells were treated with SB415286 as described in A, and Snail expression was assessed by immunofluorescence.

GSK-3α and GSK-3β isoforms serve redundant functions in epithelial cells, and that mice deficient for both GSK-3α and GSK-3β expression will exhibit prominent defects in epithelial structure and function.

The identification of NFκB as a novel target of GSK-3 activity that is relevant to the EMT is of considerable interest in light of a recent report that established NFκB as a central mediator of the EMT (Huber et al., 2004). However, this study did not define transcriptional targets of NFκB that are important for the EMT. Clearly, Snail is a prime candidate for such an NFκB target.

Although we identify NFκB and Snail as novel targets of GSK-3 activity that are relevant to the EMT, it is likely that multiple GSK-3 substrates participate in the regulation of epithelial structure. Upon its phosphorylation by GSK-3, β-catenin, a protein that has been implicated in mesenchymal transitions (Kim et al., 2002; Liebner et al., 2004), is degraded by the proteosome pathway, resulting in its inability to stimulate TCF/LEF transcription factors (Beals et al., 1997). TCF/LEF can induce the expression of genes that influence the EMT, such as vimentin (Gilles et al., 2003), the levels of which are elevated in epithelial cells treated with the GSK-3 inhibitor (Fig. 1 A). Thus, multiple signaling pathways that control the EMT are likely to be regulated by GSK-3, substantiating our hypothesis that this kinase is a central regulator of epithelial structure and function.

It is important to mention that Snail was recently identified as a direct target of GSK-3 kinase activity (Zhou et al., 2004), and the two GSK-3 phosphorylation sites identified were shown to influence Snail protein stability and localization in tumor cells. In contrast, the present study indicates that, in epithelial cells, GSK-3 impedes Snail transcription. The data presented here provide one explanation for why epithelial cells, which are characterized by high basal GSK-3 activity (Papkoff and Aikawa, 1998; Murray et al., 1999), express negligible levels of Snail mRNA (Fig. 2; Domínguez et al., 2003; Peinado et al., 2003). The combined abilities of GSK-3 to block Snail transcription, promote Snail degradation, and prevent Snail nuclear localization indicate that this kinase plays a central role in regulating Snail expression. Future studies addressing the importance of posttranslational control of Snail by GSK-3 in epithelial cells will be crucial for establishing the relevance of this mode of regulation for the EMT.

GSK-3 is a central target for the development of therapeutics because it has been implicated in numerous pathologies, including diabetes and neurologic disorders (Woodgett, 2001). Our findings suggest that this kinase is active in epithelial cells for a very important reason, namely to prevent the acquisition of a mesenchymal phenotype. Because reduced E-cadherin expression has been reported to be characteristic of some malignant tumors (Bringuier et al., 1993; Hirohashi, 1998; Kowalski et al., 2003), our findings indicate that inhibition of GSK-3, an enzyme that maintains E-cadherin expression, will not prove to be a reasonable therapeutic strategy because it has the potential to alter epithelial function significantly.

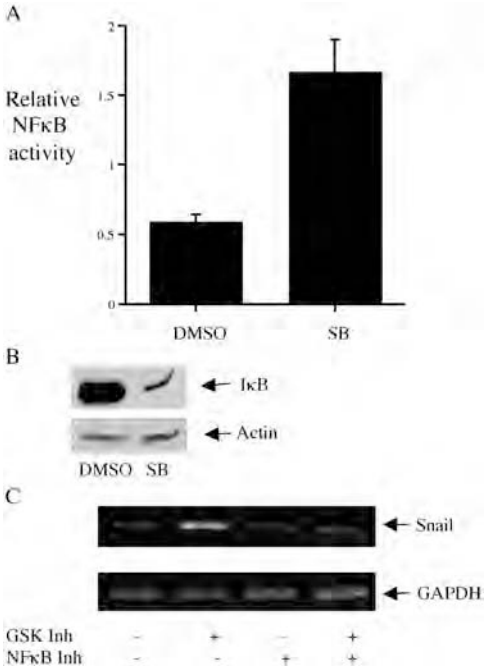

Figure 3. **GSK-3 inhibits NFκB, an activator of Snail transcription.** (A) HaCaT cells were transfected transiently with a luciferase construct driven by NFκB binding sites in addition to a control renilla luciferase reporter gene. These transfectants were then incubated with DMSO or SB415286, as described for Fig. 2, for 15 h. Data are reported as relative luciferase activity (normalized to renilla luciferase activity), ± SD, and are representative of two independent experiments. (B) MCF10A cells were incubated with DMSO or SB415286 as described for Fig. 2. Equivalent amounts of total cellular protein were extracted from these cells, subjected to SDS-PAGE, and immunoblotted with an IκB- or actin-specific antibody. Results are representative of those obtained in two independent experiments. (C) MCF10A cells were incubated for 2 h with an NFκB-specific inhibitor (SN50; Calbiochem) or control peptide (SN50M; Calbiochem). These cells were then incubated with DMSO or 25 μM SB415286. RNA was isolated after 15 h, and the levels of Snail and GAPDH were determined by RT-PCR. Similar results were obtained in three independent experiments.

Materials and methods

Cells

HaCat and HeLa cells were provided by A. Toker (Beth Israel Deaconess Medical Center, Boston, MA). MCF10A cells were provided by J. Brugge (Harvard Medical School, Boston, MA).

Analysis of protein expression

Cells were extracted in RIPA buffer. Equivalent amounts of total cellular protein extracted from these cells were subjected to SDS-PAGE and transferred to nitrocellulose. The antibodies used for immunoblotting were as follows: GSK-3 monoclonal antibody (Upstate Biotechnology), rabbit anti–cyclin D1 (Santa Cruz Biotechnology, Inc.), rabbit anti–Stat-1 (Santa Cruz Biotechnology, Inc.), mouse anti–E-cadherin (Santa Cruz Biotechnology, Inc.), rabbit antivimentin (Santa Cruz Biotechnology, Inc.), mouse anti–IκB α (Cell Signaling), and HRP-conjugated secondary antibodies (Pierce Chemical Co.).

Luciferase assays

HaCaT cells were cotransfected transiently using Lipofectin (Life Technologies) with a Snail promoter luciferase reporter construct (composed of human snail promoter sequences −869 to 59) and a control β-galactosidase reporter construct (pCS2-(n)-βgal; Promega). For NFκB studies, HaCaT cells were transfected with a luciferase construct controlled by sequential NFκB binding sites (pNF-κB-Luc; CLONTECH Laboratories, Inc.) and a control renilla luciferase construct (pRL-TK renilla luciferase; Promega). After incubation for 12 h, cells were treated with the indicated drugs (Fig. 3 A) for an additional 15 h. Luciferase and β-galactosidase activities were measured according to the manufacturer's instructions (Promega) using a luminometer and a UV/Vis spectrophotometer, respectively. Data are reported (Fig. 3 A) as the mean (± SD) relative promoter activity obtained from triplicate wells.

Analysis of mRNA expression

mRNA was purified from the indicated cell lines using the RNEasy kit (QIAGEN) according to the manufacturer's recommended protocol. 2 μg RNA was added to RT-PCR reactions containing the indicated primers at a concentration of 0.6 μM. After a 55°C/30-min reverse transcription step, Snail mRNA was PCR amplified in 32 cycles for 1 min at each of the following temperatures: 94°, 62°, and 72°C. Likewise, E-cadherin mRNA was PCR amplified in 25 cycles for 1 min at each of the following temperatures: 94°, 55°, and 72°C. PCR products were analyzed on 1% agarose gels. The sequences of amplification primers were as follows: Snail forward, GGGCAGGTATGGAGAGGAAGA; Snail reverse, TTCTTCTGCGCTACTGCTGCG; E-cadherin forward, CAGCACGTACACAGCCCTAA; E-cadherin reverse, GCTGGCTCAAGTCAAAGTCC; GAPDH forward, CCTGGCCAAGGTCATCCATGAC; and GAPDH reverse, CATGTAGGCCATGAGGTCCACCAC.

siRNA experiments

GSK-3α, GSK-3β, and control siRNA pools were designed and synthesized by Upstate Biotechnology. Cells at 60% confluence were transfected in penicillin/streptomycin-free medium with 20 μM of the indicated siRNA using TKO lipid (Mirus) according to the manufacturer's recommended protocol. Cells were harvested 48 h after transfection.

Immunofluorescence

Cells were fixed in 2% PFA, permeabilized in 0.2% Triton X-100 and 1 mM EGTA, blocked for 30 min in 1% albumin/5% donkey serum, and incubated overnight at 4°C with a Snail-specific (Domínguez et al., 2003) or control rabbit serum in blocking buffer (at a 1:20 dilution). The cells were then incubated with a fluorescein-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories) in blocking buffer (at a 1:150 dilution) for 30 min. Snail expression was analyzed using an inverted fluorescent microscope (Diaphot 300; Nikon).

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BRIEF REPORT

Autocrine semaphorin3A stimulates alpha2 beta1 integrin expression/function in breast tumor cells

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Abstract The axon repulsion factor semaphorin3A (SEMA3A) and its receptor neuropilin-1 (NP-1) are expressed in breast tumor cells, and function as suppressors of tumor cell migration. Based on the knowledge that both SEMA3A and the $\alpha2\beta1$ integrin suppress breast tumor cell migration, we studied the impact of SEMA3A signaling on $\alpha2\beta1$ integrin expression/function. The incubation of breast tumor cells with SEMA3A increased $\alpha2$ and $\beta1$ integrin levels, and stimulated tumor cell adhesion to the $\alpha2\beta1$-binding matrix protein collagen I. Conversely, reducing SEMA3A expression in breast tumor cells decreased $\alpha2\beta1$ levels and collagen adhesion. The ability of SEMA3A to increase tumor cell adhesion to collagen was dependent on both the SEMA3A receptor NP-1 and the glycogen synthase kinase-3. The incubation of breast tumor cells with SEMA3A disrupted the actin cytoskeleton, and reduced both tumor cell migratory and invasive behavior. Importantly, using an $\alpha2\beta1$-neutralizing antibody, we demonstrated that SEMA3A suppression of tumor cell migration is dependent on $\alpha2\beta1$. Our studies indicate that expression of the $\alpha2\beta1$ integrin, a suppressor of metastatic breast tumor growth, is stimulated in breast tumor cells by an autocrine SEMA3A pathway.

Keywords Semaphorin3A · Neuropilin-1 · Integrin · Migration · Breast tumor

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Introduction

The axon repulsion factor semaphorin3A (SEMA3A) promotes growth cone collapse by binding to its receptor, neuropilin-1 (NP-1) [1, 2]. Interestingly, SEMA3A and NP-1 are also expressed in endothelial cells, and serve as endogenous suppressors of integrin activity [3, 4]. Previously, our laboratory made the exciting observation that breast carcinoma cells support an autocrine signaling pathway defined by SEMA3A and NP-1 that represses cell migration [5]. However, the targets of SEMA3A signaling that impede breast tumor cell migration, and the impact of SEMA3A signaling on integrin activity in tumor cells remains unclear.

Significant evidence exists that $\alpha2\beta1$, an integrin that binds to collagen and in some cells to laminin-1 [6], is a differentiation marker for breast epithelial and tumor cells. $\alpha2$ integrin knockout mice exhibit impaired mammary gland branching morphogenesis [7]. Transgenic mice expressing a $\beta1$ integrin mutant protein lacking the extracellular domain have under-developed mammary glands with defects in epithelial differentiation [8, 9]. Finally, the degree of differentiation of primary ductal breast tumors correlates positively with $\alpha2\beta1$ integrin expression levels [10–12].

During their metastatic progression, tumor cells acquire the ability to migrate and invade tissue. Although a baseline level of the $\alpha2\beta1$ integrin is important for breast tumor cell migration and tumor growth [13, 14], excessive $\alpha2\beta1$ integrin expression is inhibitory for tumor cell migration [15, 16]. It has been demonstrated that breast tumor cell motility is increased upon reducing $\alpha2\beta1$ integrin expression levels in breast tumor cells [15]. Furthermore, the forced expression of $\alpha2\beta1$ in an $\alpha2\beta1$ integrin-deficient breast tumor cell line impairs cell motility and

tumorigenesis [16]. Finally, $\alpha2\beta1$ integrin expression is significantly reduced in metastatic relative to non-metastatic ductal breast tumors [11, 12]. Collectively, these findings suggest that the $\alpha2\beta1$ integrin can impede breast tumor metastatic progression by suppressing cell motility. These studies stress the importance of identifying endogenous proteins in breast tumor cells that regulate $\alpha2\beta1$ integrin expression. In the current work, we define an ability of autocrine SEMA3A to stimulate $\alpha2\beta1$ integrin expression in breast tumor cells, resulting in impaired tumor cell migration.

Materials and methods

Cell culture and reagents

MDA-MB-231 cells were obtained from the Duke Comprehensive Cancer Center Cell Repository, and cultured in low glucose-DMEM/5% FBS. SUM159 cells were kindly provided by Dr. Stephen Ethier, and cultured in DMEM/F12 (1:1)/5% FBS. Recombinant human semaphorin-3A/Fc, recombinant human IgG_1 Fc and neuropilin-1-neutralizing antibody (MAB566) were obtained from R and D Systems, Inc. (Minneapolis, MN). Bovine collagen type I and Fibronectin were purchased from BD Biosciences (Bedford, MA). Bovine serum albumin was purchased from Sigma (Milwaukee, WI). Hβ1.1 (mouse anti-$\beta1$ integrin), HUTS-4 (mouse anti-$\beta1$ integrin, active conformation), BHα2.1 (mouse anti-human $\alpha2\beta1$ integrin, neutralizing), rabbit anti-human integrin $\alpha2$ (CD49b) and rabbit anti-GSK-3 were purchased from Chemicon International (Temecula, CA). Donkey anti-rabbit $(Fab)_2$ and anti-mouse $(Fab)_2$ antibodies conjugated with horseradish peroxidase were from Jackson ImmunoResearch Labs (West Grove, PA). The sources of other antibodies were as follows: rabbit anti-phospho-GSK-3β (Ser9) (Cell Signaling Technology; Danvers, MA), mouse anti-β-actin (Sigma; Milwaukee, WI), rabbit anti-human SEMA3A (ECM Biosciences; Versailles, KY), isotype control antibody (mouse IgG1, Jackson ImmunoResearch Labs, West Grove, PA). Western lightning chemiluminescence reagent was from PerkinElmer (Boston, MA). GSK-3 inhibitor SB415286 was obtained from Biomol (Plymouth Meeting, PA).

Adhesion assay

Serum-starved cells (10,000) were detached for 5 min with 0.25% trypsin and resuspended in serum-free medium containing the indicated stimuli and/or antibodies. These cells were immediately plated in triplicate on 96-well microtiter plates (Fisher Scientific) pre-coated with bovine collagen type I (20 μg/ml) or bovine serum albumin (BSA) (20 μg/ml), and blocked with 0.5% BSA/DMEM. After 40 min at 37°C, these wells were washed with PBS, fixed with methanol for 10 min, and stained with 0.2% crystal violet containing 2% ethanol for 15 min. Cell adhesion was quantified in an ELISA reader by measuring absorbance (OD = 595 nm). Specific adhesion to collagen was determined by subtracting the mean OD595 (from triplicate wells) obtained on BSA from the mean OD595 (from triplicate wells) obtained on collagen ± standard deviation (SD). Statistical significance was determined using a Student's *t*-test, with a $P < 0.05$ being considered statistically significant.

Actin staining

MDA-MB-231 cells were grown on chamber slides (BD Bioscience Discovery Labware, Two Oak Park, Bedford, MA) to reach 70% confluence. After serum starving these cells overnight, they were incubated with rSEMA3A for 30 min. After three washes with PBS, cells were fixed in 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.20% Triton X-100/PBS for 20 min, and blocked in 5% bovine serum albumin/PBS (BSA) for 1 h. The slides were then incubated with Texas red-phalloidin (Becton Dickinson; 1:40) for 1 h, washed three times with PBS, incubated with DAPI for 3 min, and mounted in glycerol containing 0.1% propylgallate. Cells were examined by confocal microscopy (Light Microscopy Core Facility, Duke University) using a 40× objective.

Migration assay

Cell migration assays were performed as previously described [5]. In brief, serum-starved cells were seeded into the top chambers of transwell plates (Corning Inc., Acton, MA) in the presence of the indicated stimuli and/or antibodies. Culture medium containing 10% FBS was added to the bottom chambers. After 4 h, the cells on the bottom side of the membrane were fixed and stained with 0.2% crystal violet. The number of cells on the bottom side of the membrane were counted on an inverted microscope using a 20× objective. The mean number of migrated cells from triplicate wells ± standard deviation (SD) was determined.

Invasion assay

MDA-MB-231 cells (80% confluence) were serum starved overnight. These cells (1×10^5) were placed in the upper chamber of transwells that had been coated with Matrigel (Becton-Dickinson; 5 μg/well) and blocked with BSA (250 μg/ml)/DMEM. The ability of these cells to invade Matrigel and migrate toward 5% FBS-containing medium in the bottom chamber was measured. After 5 h, cells adhering

to the bottom side of the membrane were fixed, stained with crystal violet, and counted as described for the migration assay.

Imaging migratory cells in transwells

Cells were imaged using a Leica DMI4000 B (Leica Microsystems Inc., Bannockburn, IL) inverted microscope using the bright field setting. The images were captured with a Retiga EXi camera and a Sony progressive scan interline CCD sensor (Q imaging, Burvinab, BC, Canada). Images were displayed using Simplepci software, version 6.1.2 (Compix Inc, Sewickley, PA) on a Dell Optiplex GX620 computer (Dell Roundrock, TX).

Immunoblotting and immunoprecipitation

Proteins were extracted on ice from cells (80–90% confluence) in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, pH 7.8) containing proteinase inhibitor cocktail (Roche, Nutley, NJ). After clarification at 14,000 rpm/min at 4°C, protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc, Hercules, CA). Equivalent amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with primary and secondary antibodies according to the manufacturer's recommended conditions. For immunoprecipitations, antibody (2 μg) was added to protein lysate (50 μg) and incubated at 4°C with shaking for 2 h. Protein A/G plus agarose was added to each sample, incubated for 1 h at 4°C, and washed with protein lysis buffer.

Small hairpin RNAs (shRNA)

The complementary oligonucleotides to generate the shRNA against human SEMA3A were designed using BLOCK-iT^{TM} RNAi Designer from Invitrogen (Carlsbad, CA). Two oligo sequences were generated: shSema1 = GGGAAGAACAATGTGCCAAGG; shSema2 = GCTAGAATAGGTCAGATATGC. The double stranded (ds) oligos were subcloned into pLKOpuro1 (Yang et al. 2004). pLKOpuro1-shLuc was used as a control. pLKOpuro1-shLuc and pLKOpuro1 plasmids were a generous gift from Drs. Yang and Weinberg (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). pLKOpuro1-shLUC and pLKOpuro1-shSema were co-transfected with pCMV-VSVG and pHR′8.2ΔR (Dr. Weinberg) into 293T cells. After 48 h, the medium was collected and centrifuged for 10 min at 2,000 rpm. The supernatants were transferred into targeted cell lines, and these cell lines were selected with 1 ug/ml puromycin for 1 week. All analyses were performed using cells that had been passaged fewer than four times.

Flow cytometry

Cells were harvested with 5 mM EDTA/PBS, and incubated on ice for 30 min with an $\alpha2\beta1$-specific or isotype control IgG. After washing with PBS, these cells were incubated on ice for 30 min with FITC-conjugated goat anti-mouse IgG. Bound antibodies were detected using a Facscalibur flow cytometer (Becton Dickinson).

Reverse transcription PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription and PCR amplification were performed using the Superscript One Step RT-PCR Kit (Invitrogen, Carlsbad, CA). The following primers were used: $\beta1$ integrin (209 bp): 5′-catctgcgag tgtggtgtct-3′ (forward), 5′-ggggtaatttgtcccgactt-3′ (reverse); $\alpha2$ integrin (183 bp): 5′-gggcattgaaaacactcgat-3′ (forward), 5′-tcggatcccaagattttctg-3′ (reverse); β-actin (234 bp): 5′-gg acttcgagcaagagatgg-3′ (forward), 5′-agcactgtgttggcgtacag-3′ (reverse). The RT-PCR conditions were as follows: 50°C for 50 min, 95°C for 2 min, followed by 29 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min. A final 72°C extension for 3 min was performed. PCR products were size-fractionated on a 1.5% agarose gel, and DNA bands were visualized using ethidium bromide.

Results and discussion

Both SEMA3A [5] and the $\alpha2\beta1$ integrin [15, 16] have been independently implicated in the suppression of breast tumor cell migration, and SEMA3A reduces integrin activity in endothelial cells [4]. In the current study, we investigated SEMA3A regulation of the $\alpha2\beta1$ integrin in breast tumor cells. The incubation of MDA-MB-231 cells with a recombinant human SEMA3A-Fc fusion protein (rSEMA3A) increased $\alpha2$ and $\beta1$ subunit mRNA levels, but did not impact β-actin mRNA levels (Fig. 1a). In addition, rSEMA3A increased the levels in MDA-MB-231 cells of $\alpha2$, $\beta1$, and activated $\beta1$ protein (Fig. 1b). As evidence that this activity was not an artifact of the Fc fusion domain of this recombinant protein, the incubation of these cells with a human Fc protein did not impact expression of these integrin subunits (data not shown). $\alpha2$ and $\beta1$ integrin expression levels were increased as early as 30 min after the addition of rSEMA3A (Fig. 1b), and the ability of rSEMA3A to increase $\beta1$ integrin expression was concentration-dependent (Fig. 1c). Finally, $\alpha2\beta1$ integrin heterodimer expression was increased in rSEMA3A-pulsed as compared to control cells, as assessed by co-immunoprecipitation of the integrin subunits (Fig. 1d). In contrast, rSEMA3A did not increase the expression of $\alpha5\beta1$ integrin, as assessed by

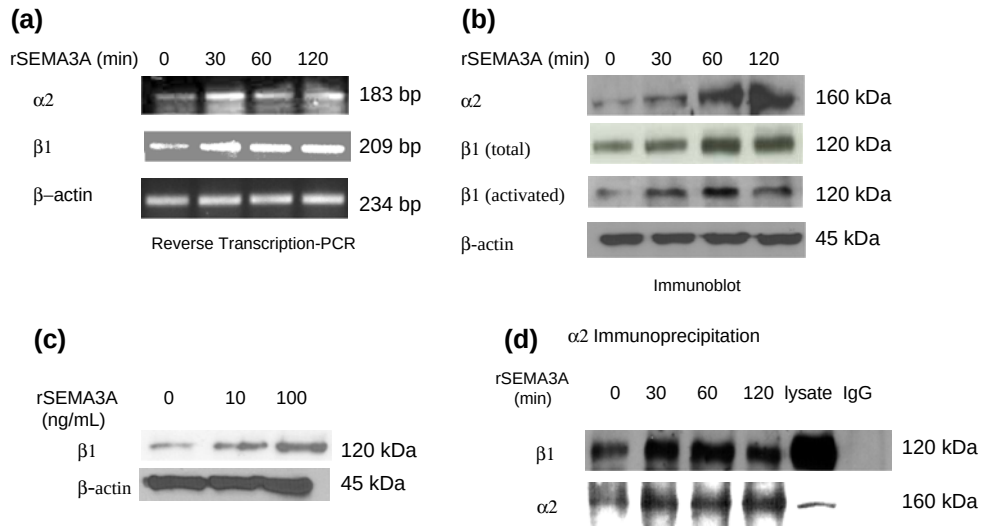

Fig. 1 SEMA3A increases $\alpha 2\beta 1$ integrin mRNA and protein expression levels in breast carcinoma cells. (**a** and **b**) MDA-MB-231 cells were grown to 80–90% confluence and serum-starved overnight. These cells were incubated with recombinant SEMA3A (rSEMA3A, 100 ng/ml) for the indicated times. (**a**) Total RNA was isolated, and $\alpha 2$, $\beta 1$ integrin and β-actin-specific reverse transcription PCR reactions were performed. PCR products were analyzed on a 1.5% agarose gel. (**b**) Total cellular proteins were extracted from these cells, and equivalent amounts were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies specific for $\alpha 2$, $\beta 1$, activated $\beta 1$, or β-actin, followed by the appropriate species secondary antibody. (**c**) MDA-MB-231 cells were grown to 90% confluence and serum-starved overnight. These cells were detached with trypsin, washed with serum-free medium, and plated on collagen-coated dishes in the absence or presence of rSEMA3A at the indicated concentration. After 40 min, total cellular proteins were extracted from these cells, and equivalent amounts of protein were subjected to SDS-PAGE. These proteins were transferred to nitrocellulose and probed with antibodies specific for $\beta 1$ integrin or β-actin, followed by the appropriate secondary antibody. (**d**) MDA-MB-231 cells were incubated with rSEMA3A for the indicated times as described in (**a**). $\alpha 2$ integrin (or isotype control IgG) immunoprecipitations were performed on equivalent amounts of total cellular protein extracted from the cells. These immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a $\beta 1$ or $\alpha 2$ integrin antibody, followed by the appropriate secondary antibody

co-immunoprecipitation of the integrin subunits (data not shown). Confirming our results in MDA-MB-231 cells, we observed an ability of rSEMA3A to increase $\alpha 2\beta 1$ integrin expression levels in SUM159 breast tumor cells (data not shown). These studies indicate that SEMA3A increases $\alpha 2\beta 1$ integrin expression levels in breast tumor cells.

We next examined the effect of incubating breast tumor cells with recombinant SEMA3A on their ability to adhere to the $\alpha 2\beta 1$-binding matrix protein collagen I. As shown in Fig. 2a and b, the incubation of either MDA-MB-231 or SUM159 breast tumor cells with a recombinant human SEMA3A-Fc fusion protein (rSEMA3A) significantly increased their adhesion to collagen I, with peak activity observed at 100 ng/ml. As a control, we showed that rSEMA3A did not influence the adhesion of these tumor cells to bovine serum albumin (BSA). We next examined if rSEMA3A impacts collagen adhesion in an $\alpha 2\beta 1$ integrin-dependent manner. As shown in Fig. 2c, the incubation of MDA-MB-231 cells with an $\alpha 2\beta 1$ integrin-neutralizing antibody suppressed the ability of rSEMA3A to increase cell adhesion to collagen. The incubation of these tumor cells with rSEMA3A in medium containing EDTA did not increase their adhesion to collagen, indicating the cation-dependence of this SEMA3A activity (Fig. 2d).

Our previous studies demonstrated that SEMA3A is expressed in breast tumor cell lines and primary tumors [5]. Accordingly, we next investigated if endogenous SEMA3A impacts the adhesion of breast tumor cells to collagen. MDA-MB-231 cells were infected with lentiviruses expressing either of two SEMA3A-specific small hairpin RNAs (shRNAs) or a control luciferase shRNA (shLuc RNA). SEMA3A expression was reduced in cells infected with these SEMA3A shRNA-expressing vectors as compared to that observed in shLuc RNA-expressing cells (Fig. 3a). Importantly, SEMA3A shRNA-expressing cells exhibited a significantly reduced ability to bind to collagen I as compared to control infectants (Fig. 3b). Moreover, reducing SEMA3A expression in these cells decreased the levels of $\alpha 2$ and $\beta 1$ integrin subunits (Fig. 3c). Collectively, these results indicate that autocrine SEMA3A stimulates breast tumor cell adhesion to collagen by increasing $\alpha 2\beta 1$ expression.

Based on our previous demonstration that the SEMA3A receptor NP-1 is expressed in breast tumor cells [5], we examined a role for NP-1 in regulating tumor cell adhesion to collagen. As shown in Fig. 4a, the ability of rSEMA3A to stimulate MDA-MB-231 cell adhesion to collagen was inhibited by incubating these cells with a NP-1-neutralizing

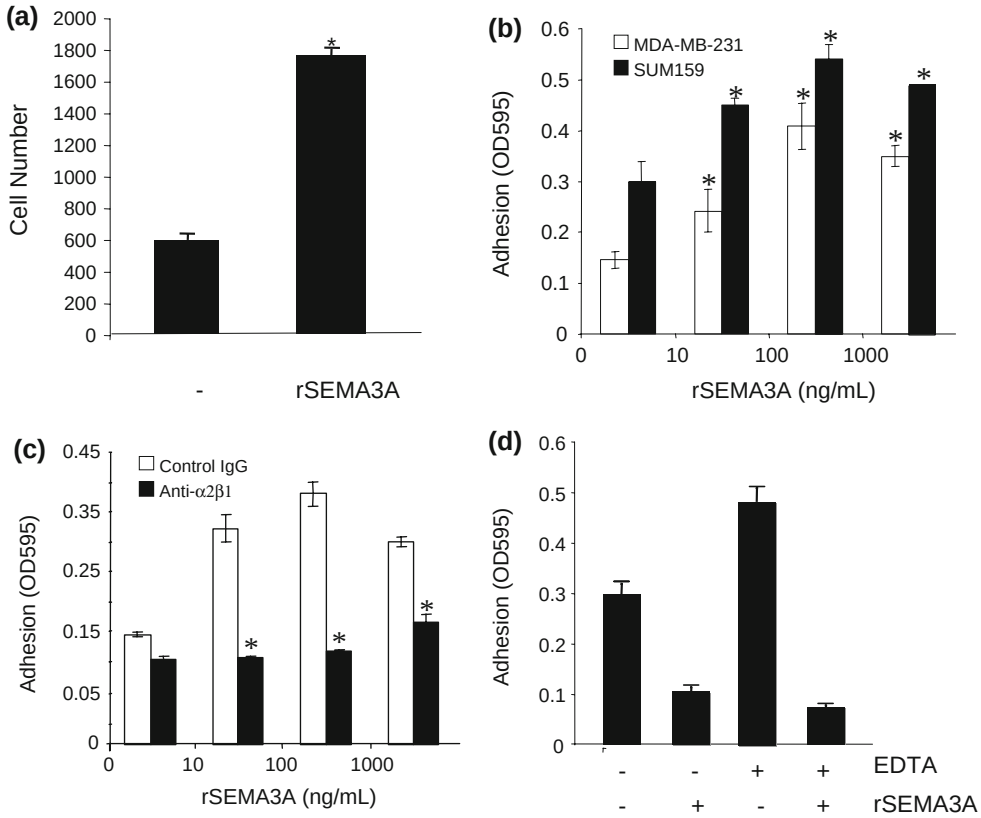

Fig. 2 Incubation of breast tumor cell lines with recombinant SEMA3A increases their adhesion to collagen I in an $\alpha 2\beta 1$ integrin-dependent manner. (**a**) MDA-MB-231 cells were grown to 80–90% confluence and serum-starved overnight. Cells were resuspended in serum-free medium in the absence or presence of rSEMA3A (100 ng/ml), and plated on 96-well microtiter plates pre-coated with bovine collagen type I (20 μg/ml). After 40 min, non-adherent cells were removed, bound cells were fixed with methanol, and adherent cells were stained with crystal violet. Adherent cells were counted by phase contrast microscopy using a 10× objective. Data is presented as the mean number of cells per well from each of three wells (±SD). (**b**) MDA-MB-231 (white bar) and SUM159 (black bar) cells were grown to 80–90% confluence and serum-starved overnight. Cells were resuspended in serum-free medium in the absence or presence of rSEMA3A (0, 10, 100, 1,000 ng/ml), and plated on 96-well microtiter plates pre-coated with bovine collagen type I (20 ug/ml) or bovine serum albumin (20 μg/ml). After 40 min, non-adherent cells were removed, adherent cells were fixed with methanol and stained with crystal violet, and cells were permealized with 1% SDS. Adhesion was quantified in an ELISA reader by measuring absorbance at 595 nm (OD595). Data are presented as the difference between the mean OD595 on BSA (from triplicate wells) and the mean OD595 on collagen (from triplicate wells) ± standard deviation (SD). $^*P < 0.05$ in a student's *t*-test. Similar results were obtained in two independent experiments. (**c**) The adhesion of MDA-MB-231 cells to collagen was measured as described in (**b**) except that cells were resuspended in serum-free medium in the presence or absence of rSEMA3A in addition to an isotype control IgG or $\alpha 2\beta 1$ integrin-neutralizing antibody (anti-$\alpha 2\beta 1$) (1 ug/ml). $^*P < 0.05$ in a student's *t*-test. (**d**) Serum-starved MDA-MB-231 cells were pre-incubated in EDTA (5 mM)-containing or control medium for 30 min. These cells were then incubated in the presence or absence of rSEMA3A (100 ng/ml) in 96 well plates coated with collagen or BSA, as described for (**b**). Data are reported as indicated for (**b**). $^*P < 0.05$ in a student's *t*-test

antibody. Because SEMA3A stimulates the serine/threonine kinase GSK-3 by binding to NP-1 in neurons [17], we next examined a role for this kinase in regulating $\alpha 2\beta 1$ integrin expression in breast tumor cells. The incubation of MDA-MB-231 cells with rSEMA3A stimulated GSK-3 activity, as evidenced by reduced levels of phosphorylated GSK-3 (the inactive form of GSK-3) in rSEMA3A-treated compared to control cells (Fig. 4b). Conversely, we observed increased levels of phosphorylated GSK-3 in SEMA3A shRNA-expressing as compared to control MDA-MB-231 cells (Fig. 4b). As evidence that GSK-3 is important for SEMA3A regulation of cell adhesion, the ability of rSEMA3A to increase MDA-MB-231 cell adhesion to collagen was inhibited in cells that had been pre-incubated with SB415286, a highly-specific small molecule GSK-3 inhibitor [18] (Fig. 4c). Furthermore, rSEMA3A did not increase $\beta 1$, activated $\beta 1$, or $\alpha 2\beta 1$ expression levels in MDA-MB-231 cells incubated with SB415286 (Fig. 4d). These data indicate that SEMA3A induction of $\alpha 2\beta 1$ integrin expression and adhesive function is dependent on its activation of GSK-3. Although previous studies defined a role for GSK-3 in integrin recycling [19], our work indicates that the SEMA3A/GSK-3 signaling axis influences $\alpha 2\beta 1$ integrin expression levels in tumor cells. Further work is needed to determine the level at which GSK-3 regulates $\alpha 2\beta 1$ integrin expression,

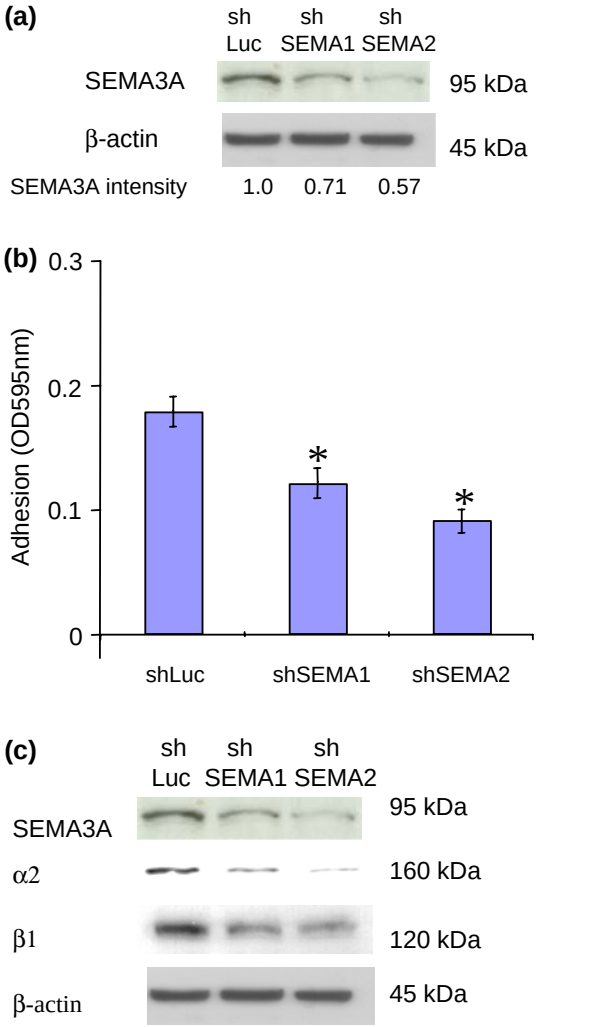

Fig. 3 Endogenous SEMA3A in breast tumor cells promotes $\alpha 2\beta 1$ expression and collagen adhesion. (**a**) MDA-MB-231 cells were infected with lentiviruses expressing shSEMA3A RNA#1 (shSema1), shSEMA3A RNA#2 (shSema2), or shLuciferase RNA (shLuc). Equivalent amounts of protein extracted from these cells (90% confluence) were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with a SEMA3A or β-actin antibody, followed by the appropriate secondary antibody. The intensity of SEMA3A bands was quantified by densitometry using ImageJ software (NIH). (**b**) MDA-MB-231 subclones expressing shSEMA3A RNAs (shSema1 and shSema2) or shLuc RNA were grown to confluence, resuspended in serum-free medium, and plated on 96-well microtitre plates coated with bovine collagen type I (20 ug/ml). After 40 min, collagen adhesion was measured as described in Fig. 2b. Data are presented as the difference between the mean OD595 on BSA (from triplicate wells) and the mean OD595 on collagen (from triplicate wells) ($\pm$SD). $^*P < 0.05$ in a student's t-test. Similar results were obtained in two independent experiments. (**c**) Total cellular proteins were extracted from MDA-MB-231 subclones expressing shSEMA3A RNAs (shSema1 and shSema2) or shLuciferase (shLuc). Equivalent amounts of protein were subjected to SDS-PAGE and immunoblotted with a SEMA3A, $\alpha 2$, $\beta 1$, or β-actin antibody, followed by the appropriate species secondary antibody

and to identify the target of GSK-3 kinase activity that regulates expression of this integrin.

During their metastatic progression, tumor cells acquire the ability to migrate. Several studies indicate that the acquisition of an intermediate cell adhesive strength promotes optimal cell migration [20, 21]. Accordingly, while a baseline level of $\alpha 2\beta 1$ is essential for cell migration, cell migration is suppressed by $\alpha 2\beta 1$ integrin expression levels beyond this baseline [15, 16]. Likewise, reducing $\alpha 2\beta 1$ integrin expression levels below this baseline inhibits cell migration [15]. Considering that SEMA3A is an endogenous suppressor of breast tumor cell migration [5], we sought to determine if SEMA3A suppresses breast tumor cell migration by stimulating the $\alpha 2\beta 1$ integrin. Based on the knowledge that MDA-MB-231 cells are highly migratory [5], we predicted that these cells express an intermediate concentration of $\alpha 2\beta 1$ optimal for migration. We hypothesized that significant increases or decreases in $\alpha 2\beta 1$ expression or activity in these cells would impair cell migration. First, we assessed the effect of rSEMA3A on the actin cytoskeleton. MDA-MB-231 cells were incubated for 30 min with or without rSEMA3A in serum-free medium, and F-actin levels were assessed by phalloidin staining. As shown in Fig. 5a, actin filaments were abundant in control MDA-MB-231 cells, and lamellipodia were observed at the leading migration edge. In contrast, MDA-MB-231 cells incubated with rSEMA3A showed reduced phalloidin staining, exhibited a less organized actin filament network, and lacked visible lamellipodia. This ability of rSEMA3A to disrupt the actin cytoskeleton suggested that rSEMA3A may reduce tumor cell migration. To examine this question, we assessed the ability of control and rSEMA3A-incubated MDA-MB-231 cells to migrate toward serum-containing medium in a transwell assay. As shown in Fig. 5b and c, rSEMA3A significantly reduced MDA-MB-231 cell migration. Importantly, rSEMA3A also decreased the invasive potential of MDA-MB-231 cells, as assessed using Matrigel-coated transwells (Fig. 5c).

Based on our demonstration that rSEMA3A increases the expression in breast tumor cells of $\alpha 2\beta 1$, an integrin that can reduce tumor cell migration [15, 16], we next investigated the importance of $\alpha 2\beta 1$ for SEMA3A regulation of breast tumor cell migration. Serum-starved MDA-MB-231 cells were incubated with control medium or rSEMA3A-containing medium in the presence of a control or an $\alpha 2\beta 1$ integrin-neutralizing antibody. The ability of these cells to migrate in a transwell assay was then investigated. The incubation of MDA-MB-231 cells with an $\alpha 2\beta 1$-neutralizing antibody reduced cell migration, consistent with the idea that these cells are reliant on

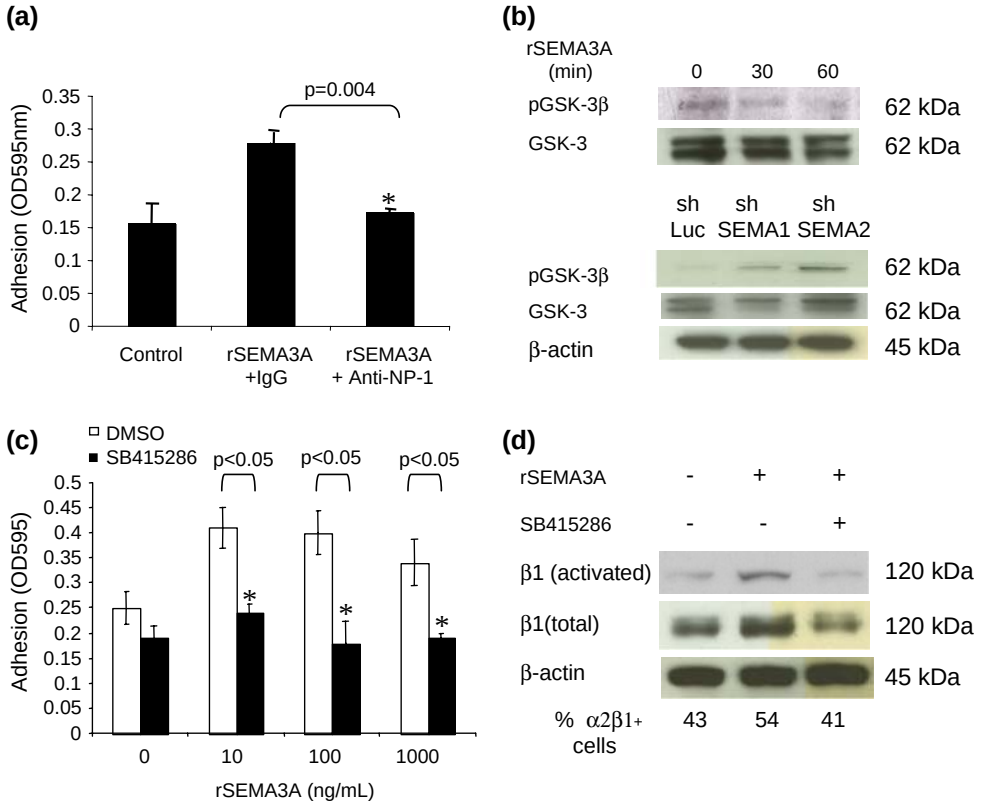

Fig. 4 The ability of SEMA3A to increase breast carcinoma adhesion to collagen is dependent on NP-1 and GSK-3. (**a**) MDA-MB-231 cells were incubated with a NP-1-neutralizing (anti-NP-1) or isotype control (control Ig) antibody (1 μg/ml) in the presence or absence of the indicated concentration of rSEMA3A. The ability of these cells to adhere to collagen was assessed as described for Fig. 2b. (**b**) (Top panel) MDA-MB-231, cells were grown to 80–90% confluence and serum-starved overnight. These cells were incubated with rSEMA3A at a concentration of 100 ng/ml and harvested at 0, 30, or 60 min. Equivalent amounts of total cellular protein extracted from these cells were subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted with antibodies specific for phosphorylated or total GSK-3, followed by the appropriate species HRP-conjugated secondary antibody. (Bottom panel) MDA-MB-231 cells expressing shSEMA3A RNAs (shSema1 and shSema2) or shLuc RNA were serum-starved overnight, and immunoblotted with antibodies specific for phospho-GSK-3β (Ser9), GSK-3 or β-actin, followed by the appropriate species secondary antibody. (**c**) MDA-MB-231 cells were pre-incubated with the GSK-3 inhibitor SB415286 (25 μM) or DMSO for 1 h, and then incubated with or without rSEMA3A at the indicated concentration. Relative cell adhesion to collagen was determined as described in Fig. 2b. $^*P < 0.05$ in a student's *t*-test. (**d**) MDA-MB-231 cells were grown to 80–90% confluence and serum-starved overnight. The cells were pre-treated with the GSK-3 inhibitor SB415286 (25 μM) or DMSO for 1 h, then incubated with rSEMA3A (100 ng/ml) for 40 min. For immunoblots, total cellular protein was extracted from these cells, and equivalent amounts run on an SDS polyacrylamide gel. These proteins were transferred to nitrocellulose and immunoblotted with an antibody specific for activated or total β1, followed by the appropriate species secondary antibody. As a loading control, a β-actin immunoblot was performed. Alternatively, to assess cell surface integrin expression, MDA-MB-231 cells were serum-starved overnight, detached with trypsin, and resuspended in serum-free medium containing: (1) DMSO, (2) rSEMA3A + DMSO, or (3) rSEMA3A + GSK-3 inhibitor (SB415286, 25 μM). These cells were immediately plated onto collagen-coated dishes. After 40 min, the cells were collected and incubated with an $\alpha2\beta1$ integrin or isotype control antibody, followed by a FITC conjugated secondary antibody. These cells were analyzed by flow cytometry. Data are reported as the percent $\alpha2\beta1$ integrin-positive cells, and are representative of three different trials

a baseline level of $\alpha2\beta1$ integrin to support optimal migration (Fig. 6). We also observed that the incubation of MDA-MB-231 cells with rSEMA3A significantly reduced their migration (Fig. 6). However, SEMA3A did not repress the migration of MDA-MB-231 cells incubated with an $\alpha2\beta1$ integrin-neutralizing antibody (Fig. 6). This data indicates that SEMA3A suppresses breast tumor cell migration in an $\alpha2\beta1$ integrin-dependent manner.

Collectively, our work establishes that an autocrine pathway defined by SEMA3A and NP-1 drives $\alpha2\beta1$ integrin expression and adhesive function in breast tumor cells. This finding is in agreement with previous work defining NP-1 as an adhesion-promoting molecule in endothelial cells [22]. However, our results contrast with other studies indicating that SEMA3A can repress the adhesion of other cell types to matrix proteins [4, 23–25]. These conflicting results may reflect an ability of SEMA3A

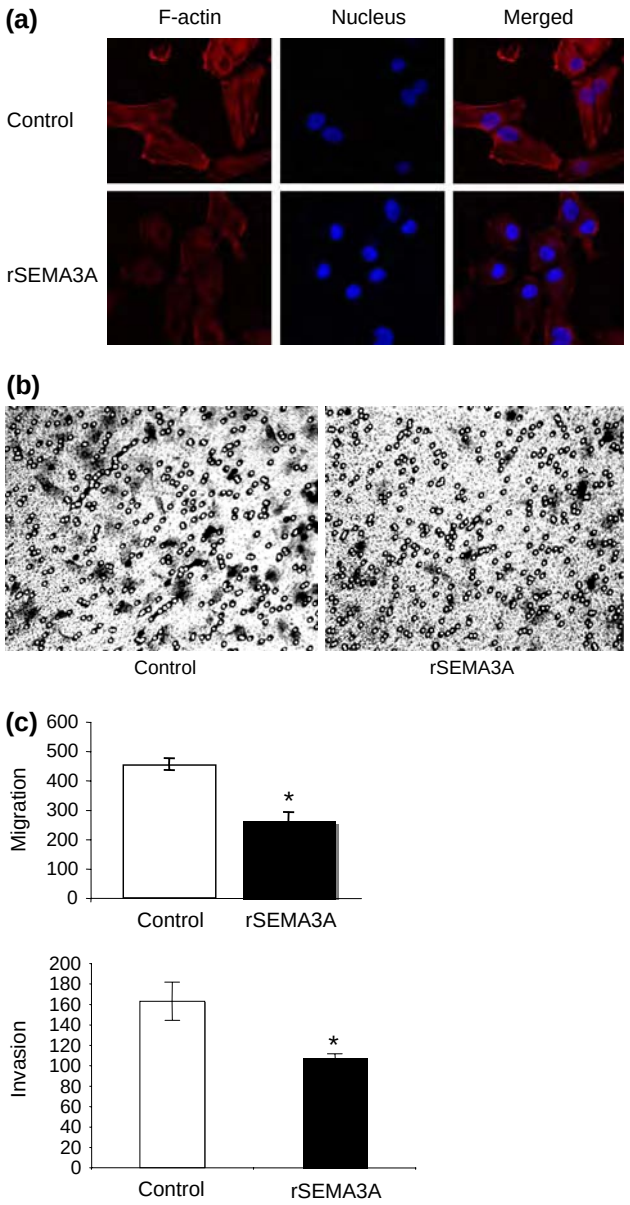

Fig. 5 rSEMA3A decreases breast tumor cell migration and invasion. (**a**) MDA-MB-231 cells were grown on chamber slides and serum-starved overnight. These cells were then incubated ± rSEMA3A in serum-free medium for 30 min. Cells were fixed, permeabilized, and incubated with Texas red-phalloidin and DAPI. These samples were visualized by confocal microscopy. (**b**) Serum-starved MDA-MB-231 cells were added to the top chambers of a collagen-coated transwell in serum-free medium ± rSEMA3A (100 ng/ml). Culture medium containing 10% FBS was added to the bottom chambers. After 4 h, the cells that had migrated to the bottom side of the membrane were fixed and stained with 0.2% crystal violet. Cells were visualized by microscopy using a 20× objective. (**c**) Serum-starved MDA-MB-231 cells were added to the top chambers of Collagen I or Matrigel-coated transwells in serum-free medium ± rSEMA3A (100 ng/mL). Culture medium containing 10% FBS was added to the bottom chambers. After 5 h, cells on the bottom of the transwell membrane were stained with crystal violet and counted as described in Fig. 2b

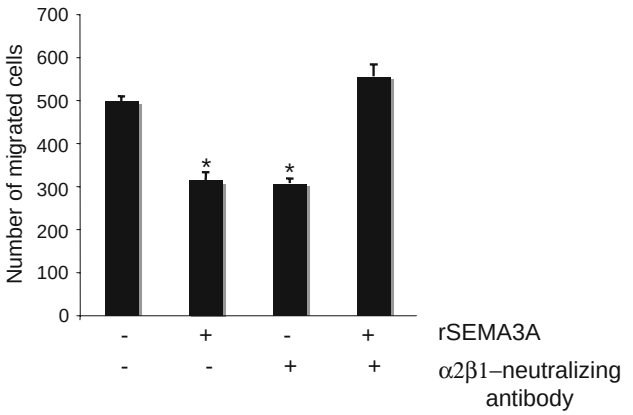

Fig. 6 rSEMA3A decreases breast tumor cell migration, but not in the presence of an $\alpha 2\beta 1$ integrin-neutralizing antibody. MDA-MB-231 cells (80–90% confluent) were serum-starved overnight. These cells were then added to the top chambers of a collagen-coated transwell in serum-free medium ± rSEMA3A (100 ng/ml) and either an $\alpha 2\beta 1$ integrin-neutralizing antibody (anti-$\alpha 2\beta 1$) or isotype control antibody (control Ig) at a concentration of 1 μg/ml. Culture medium containing 10% FBS was added to the bottom chambers. After 4 h, the cells that had migrated to the bottom side of the membrane were fixed and stained with 0.2% crystal violet. Cells were visualized and counted by microscopy using a 20× objective. The data are presented as the mean number of migrated cells ± standard deviation (SD) from three wells). $^*P < 0.05$ in a student's *t*-test

and NP-1 to differentially impact the adhesion of divergent cell types based on their expression of different SEMA3A co-receptors. For example, a soluble form of the NP-1 co-receptor L1 can convert SEMA3A growth cone collapsing activity into growth cone attraction [26].

Our previous studies established that beyond its angiogenic activities, the vascular endothelial growth factor (VEGF-A) exhibits autocrine functions in breast tumor cells [5]. We also showed that VEGF-A competes with Sema3A for NP-1 binding in breast tumor cells, thus inhibiting autocrine Sema3A signaling [5]. Based on these findings, we predict that tumor cells characterized by a high VEGF-A: Sema3A ratio will exhibit reduced $\alpha 2\beta 1$ expression levels and increased invasive behavior compared to tumor cells associated with a high Sema3A:-VEGF-A ratio, a topic of current investigation.

Several studies indicate that the $\alpha 2\beta 1$ integrin is a differentiation marker for breast tumor cells and an inhibitor of tumor growth [7–12]. Our studies are important in defining an autocrine regulator of $\alpha 2\beta 1$ integrin expression in breast tumor cells. Importantly, we also show that autocrine SEMA3A reduces cell migration by stimulating the $\alpha 2\beta 1$ integrin. Based on the knowledge that tumor metastasis is dependent on tumor cell migratory and invasive behavior, we predict that breast tumor cells that support autocrine Sema3A signaling are likely suppressed in their metastatic potential, a topic of current investigation. Our studies also suggest that the delivery of SEMA3A

to tumors may impede their metastasis by increasing $\alpha 2\beta 1$ integrin expression levels, a topic for future studies.

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SUPPORTING DATA:

Fig. 1: Detection of Snail-1 protein in breast tumor cell lines and primary breast tumors. **A.** Total cellular proteins were extracted from human mammary epithelial cells (HMEC) as well as from two metastatic human breast cancer cell lines (MDA-MB-231, DKAT). Total cellular protein (100 µg per lane) was run on an SDS polyacrylamide gel, transferred to nitrocellulose, and probed overnight with a Snail-1 monoclonal antibody[9] at a dilution of 1:200, followed by IRDye700 (Rockland Inc.)-conjugated secondary antibody. Protein bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences), and quantified by densitometry (ImageJ, NIH). **B.** Primary human breast cancer specimens were fixed in formalin and embedded in paraffin. Antigen retrieval was accomplished by boiling the samples in citrate buffer, pH 9.0. After blocking in 1% BSA, sections were incubated with the Snail-1 monoclonal antibody[9] at a dilution of 1:200 overnight (or an isotype control antibody at equal concentration). Bound antibody was detected using anti-mouse Envision (Envision System Peroxidase, DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin.

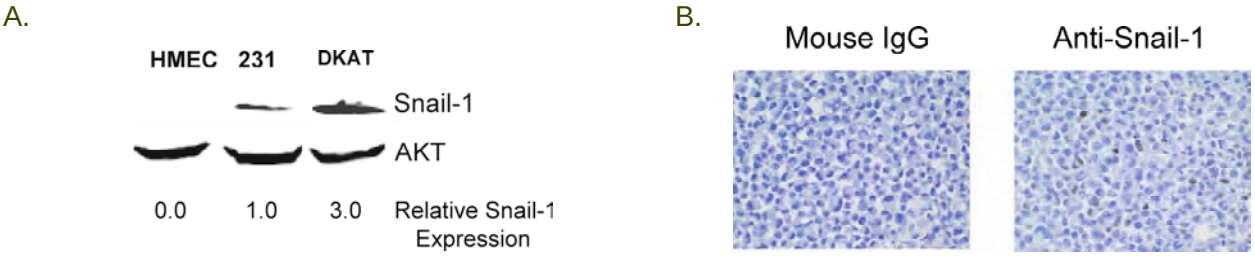

Fig. 2: Development of a Snail-1 siRNA transfection protocol for effectively reducing Snail-1 expression in MDA-MB-231 cells. **A.** MDA-MB-231 cells were transfected using Lipofectamine (Invitrogen) with a Snail-1-specific siRNA (Santa Cruz Biotechnology) or control siRNA at the indicated concentrations. After 8 hours, mRNA was harvested using TRIzol reagent (Invitrogen). Snail-1 and β-actin-specific reverse transcription-PCR was performed using our previously published protocol[10]. **B.** MDA-MB-231 cells were transfected as described above with a Snail-1-specific or control siRNA at a concentration of 50 nM. Total cellular proteins were harvested at 8 hours or 40 hours post-transfection. Equivalent amounts of protein (100 µg/lane) were loaded onto an SDS polyacrylamide gel, transferred to nitrocellulose, and probed with a Snail-1 antibody as described in Fig. 1. Densitometry (Image J, NIH) indicates that the Snail-1 siRNA-induced reduced Snail-1 protein levels by 1.6-fold at 8 hours post-transfection and by 4.9-fold at 40 hours post-transfection.

Fig. 3: Reducing Snail-1 expression in MDA-MB-231 cells increases alpha2 integrin levels. MDA-MB-231 cells were transfected with a Snail-1 or control siRNA as described in Fig. 2. After 40 hours, total cellular proteins were harvested, and equivalent amounts were loaded onto an SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose, and probed with the indicated integrin antibodies, followed by IRdye-conjugated secondary antibody using our published protocol[11]. Protein bands were detected as described for Fig. 1. Densitometry indicates a 2-fold increase in alpha2 integrin levels in Snail-1 siRNA transefctants compared to control transfectants.

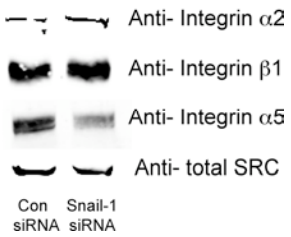

Fig. 4: Reducing Snail-1 expression in MDA-MB-231 cells increases cell adhesion to Collagen-1. MDA-MB-231 cells were transfected with Snail-1-specific or control siRNA as described for Fig. 2. After 40 hours, cells were harvested with trypsin, and their ability to adhere to Collagen-I-coated or Fibronectin-coated wells was assessed using our published protocol[11]. Relative adhesion is presented as OD595.